EXPERIMENTAL PRODUCTION OF CHAINS AND ITS GENETIC CONSEQUENCES IN THE CILIATE PROTOZOAN, COLPIDIUM CAMPYLUM (STOKES)

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I. INTRODUCTION

In 1925, Édouard and Mme. Chatton showed that chains of individuals could be produced in *Colpidium campylum* and *Colpidium colpoda* by feeding them a certain strain of the bacterium *Bacillus coli* (*"Colibacillus D"*). The present paper deals with the genetic consequences of a similar chain-formation induced in *Colpidium campylum* [2] by a strain of another bacterium (*Micrococcus sp.*, probably *sensibilis*). The present work is in part an independent corroboration of the work of the Chattons, because the author was unacquainted with their work until the essential points in which the two investigations agree had already been established.

In the present work, though not in the work of the Chattons, experimentally produced chains gave rise to a new racial type,—double animals. As a result of this, the question in the foreground of interest

[1] During the performance of most of the experimental work, the author was Fellow of the National Research Council. The author wishes to acknowledge his indebtedness to Professor H. S. Jennings for many important suggestions, particularly concerning preparation of the manuscript; and to Dr. Ruth S. Lynch for critical reading of the manuscript.

[2] Identified through the courtesy of Professor Édouard Chatton.

in this paper is: What are the genetic consequences of the experimental production of chains? In the treatment of this question, the following matters will be taken up: (1) the experimental determination of chain-formation; (2) the mode of origin of chains and their subsequent development into races of doubles; (3) the persistence and stability of the races of doubles; (4) the existence of diverse biotypes among doubles and their descendants; (5) an examination of the nature of the processes involved in these changes of hereditary characteristics in vegetative reproduction.

II. Materials and Methods

The methods employed for the cultivation of large mass cultures of *Colpidium* were given in an earlier paper (Sonneborn, 1930*a*).[3] The basic fluid was an infusion of 1.5 grams rye grains boiled for ten minutes in 100 cc. spring water, filtered and let stand for 24 hours to "ripen." Two hundred cc. of this infusion plus five boiled rye grains were put into a finger bowl 10 cm. in diameter and 4 cm. deep. This was inoculated with 10 to 15 cc. of an old culture of *C. campylum;* within 24 hours it would develop into a flourishing culture and remain so for several days. When the colpidia began to get smaller and paler, usually in two to six days, subcultures were made in the same way. Only large stock cultures were maintained in this way.

Smaller mass cultures were kept in square salt cellars and in Columbia dishes. These were begun with several drops of ripe fluid (without any rye grains) and ten to thirty colpidia; each day more ripe fluid was added until after three to five days the dish was full (about thirty drops). Subcultures were then made in the same way.

In special experiments designed to investigate carefully the factors determining chain-formation, Columbia dish cultures were made in a different way, following, in many respects, the bacteriological technique employed by Raffel (1930) in the cultivation of *Paramecium.* The standard rye infusion, as soon as filtered, was distributed in cotton-plugged test tubes, autoclaved and stored until needed. When needed, a tube was opened over a Bunsen flame and inoculated by means of a platinum needle with a pure culture of either *Achromobacter sp.,* probably *candicans,* or *Micrococcus sp.,* probably *sensibilis,* grown on beef-agar slants. The inoculated culture fluid was then pipetted into Columbia dishes, inside of Petri dishes. These, and the cotton-plugged pipettes, inside of jars, had all been heated for one hour at 150° C. in a hot-air sterilizer. Each pipette was used only once and the top of the Petri dish containing the Columbia dish was raised only enough to admit

[3] In that paper, the species was incorrectly called *Colpidium striatum.*

the pipette with culture fluid and colpidia. The colpidia had previously been washed according to the method of Parpart (1928). After such a culture was set up, it was opened only once for purposes of subculture.

In all the rest of the work, the colpidia were cultured in isolation on ground glass slides containing two concavities. Twelve of these slides were placed on a glass plate raised on glass supports in an inverted 9-inch Petri dish sealed with water at the bottom. The "ripe" rye infusion was used as cultivation medium, one drop to each concavity. Each day one *Colpidium* was placed in such a drop; 24 hours later the drop was again observed, records of reproduction and other matters of interest were made, and one of the colpidia was transferred to a fresh drop of ripe culture fluid. Each day a similar procedure was followed.

A number of other details of procedure in the isolation cultures are important: (1) The possibility of perpetuating, by daily transfers, differences in bacterial flora between different lines was avoided by collecting a small amount of fluid from each 24-hour-old culture drop and using these small drops for inoculating the culture fluid for the next day. Such cross-inoculations were performed daily in some experiments, less frequently in others. (2) The possibility of repeating daily systematic differences in the treatment of the lines compared was avoided by the following methods: (a) within each moist chamber containing 24 lines, each type of animal in a particular set of comparisons was represented by the same number of lines; (b) within each moist chamber the lines were distributed according to a plan whereby the lines of the same type or race were separated from each other and whereby the arrangement in no two moist chambers was the same; (c) the order in which the moist chambers were transferred was systematically changed from day to day. (3) The possibility of personal bias influencing the results was avoided by assigning to each line for daily identification a name that gave no indication of its genetic history. The key to these names was not consulted until the end of the experiment.

Many of the experiments were conducted at room temperature of 20°–25° C., some were run in a constant temperature chamber which only rarely exceeded the range 22°–23° C. The animals used in all the work here reported were descended from one individual and were never known to conjugate, even when efforts were made to make them do so.

III. The Determination of Chain-Formation

As already mentioned, Édouard and Mme. Chatton were able to induce the formation of chains in *Colpidium campylum* and *Colpidium colpoda* by feeding these species a particular strain of *Bacillus coli*. Other species of bacteria, other strains of *Bacillus coli*, and this strain

grown on other than a vegetable base did not induce chain-formation in *Colpidium*. These investigators thus demonstrated that the formation of chains in *Colpidium* is determined by a particular strain of bacteria grown on a vegetable base.

This result of the Chattons was confirmed by the present investigation, as will now be set forth. The rye infusion in which chains of colpidia had arisen was plated out on agar (with the help of Dr. Raffel) and the different kinds of bacteria were separately cultivated. Only two kinds of colonies could be distinguished: one yellow and rapidly growing, the other white and slowly growing. These two species were identified for me through the courtesy of Professor William W. Ford of the Department of Bacteriology, School of Hygiene and Public Health, the Johns Hopkins University. The yellow species was identified as belonging to the genus *Micrococcus* (Bergey) and corresponding closely to the species *M. sensibilis;* the white species was identified as belonging to the genus *Achromobacter* (Bergey) and corresponding closely to the species *A. candicans.*

A number of experiments indicated an increase in the frequency of chain-formation when *Micrococcus* predominated in the food supply. In a few cultures some chains were formed in fluid in which *Achromobacter* predominated; in these cultures, however, *Micrococcus* had not been excluded. In all other cultures in which chains were formed, *Micrococcus* had definitely been inoculated into the culture fluid.

Critical experiments, using the rigorous bacteriological methods described above in Section II, were performed to discover the relation of these two species of bacteria to chain-formation. On July 29, eight normal colpidia were washed, according to Parpart's (1928) method, in two steps: five washings were followed by a lapse of five hours, after which five more washings were made. Four of the eight colpidia were washed in autoclaved rye fluid inoculated with *Achromobacter;* the other four in similar fluid inoculated with *Micrococcus.* By July 31, each set of four had multiplied to form eighteen colpidia. The animals in *Micrococcus* fluid were used to establish nine cultures, each consisting of two colpidia in sixteen drops of autoclaved rye fluid inoculated with *Micrococcus.* The animals in *Achromobacter* were used to establish nine similar cultures in *Achromobacter* fluid. Counts of the number of singles and the number of chains in each of these eighteen cultures were made on August 3. As appears in Table I, not one chain was formed among 46,716 colpidia produced in the pure *Achromobacter* fluid, but 51 chains were formed among 11,912 colpidia produced in the pure *Micrococcus* fluid. The production of chains is clearly deter-

TABLE I

Comparison of the percentage of chains formed in cultures fed Micrococcus exclusively, with the percentage formed in cultures fed Achromobacter exclusively

BACTERIUM SUPPLIED		CULTURES									
		1	2	3	4	5	6	7	8	9	Total
Achromobacter	Number of Colpidia	5765	6720	5741	4849	5186	5815	4240	4852	3548	46716
	Number of Chains	0	0	0	0	0	0	0	0	0	0
	Percentage of Chains	0	0	0	0	0	0	0	0	0	0
Micrococcus	Number of Colpidia	1779	1184	1679	1354	1576	1071	1336	1224	1709	11912
	Number of Chains	21	1	1	5	2	0	6	0	15	51
	Percentage of Chains	1.18	0.08	0.06	0.37	0.13	0	0.45	0	0.88	0.43

mined by the presence of the *Micrococcus*. However, only a small proportion of the colpidia subjected to *Micrococcus* form chains. This is in striking contrast to the result reported by the Chattons with "*Colibacillus D.*" In their work, all dividing colpidia formed chains in this bacterium. The relatively small proportion of chains produced with *Micrococcus* may have been due to the fact that this bacterium was cultivated on beef agar before being introduced into the rye fluid with *Colpidium*. The Chattons, it will be recalled, showed that the chain-inducing power of *Colibacillus D* was lost when it was grown on other than vegetable bases.

In the present work the existence of additional factors in the determination of chain-formation was indicated by the considerable variation in percentage of chains formed among the nine cultures reared on pure *Micrococcus*. One of these factors may be the concentration of colpidia in the culture fluid. Although all cultures began with two colpidia in sixteen drops, there was much variation in their concentration at the end of the experiment. The mean number of animals per micropipette drop varied from 48.7 to 84.7 colpidia. The five cultures in which there were more than 70 colpidia per droplet produced, on the average, 0.54 per cent chains; the four cultures in which there were less than 60 colpidia per drop produced, on the average, 0.11 per cent chains. There is, therefore, some indication that the percentage of chains formed when fed *Micrococcus* depends to some extent on the concentration of colpidia in the fluid.

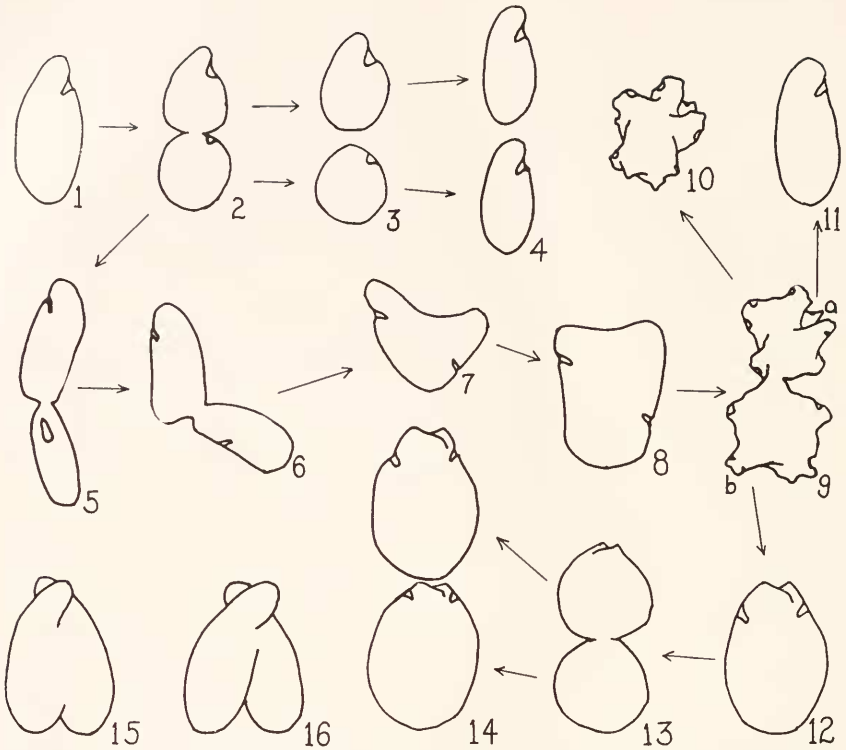
The essential point, however, is that the difference between cultures which remain normal and cultures which produce chains is due to the difference between the effects of two diets: cultures fed only *Achromobacter* remain normal; cultures fed *Micrococcus* produce a small percentage of chains. This dependence of chain-formation in *Colpidium* upon the presence of particular strains of bacteria confirms the earlier results of the Chattons. The latter investigators attempted also to induce chain-formation in other genera of ciliates with the strain of bacteria they found effective for *Colpidium*. Such attempts failed completely. The present investigator also attempted to induce chains in *Paramecium aurelia*, by feeding this species the strain of *Micrococcus* found to be effective for *Colpidium*. Like the attempts of the Chattons, this attempt also failed. It appears that *Colpidium* is a genus particularly susceptible to this type of bacterial action.

IV. Origin of Chains and Their Subsequent Development

Chains originate simply as a consequence of the development of the adult form in the two parts of a dividing individual, in the absence of

the normal separation of these two parts. A normal adult (Fig. 1) becomes constricted transversely in about the mid-region (Fig. 2). In normal fission, separation of the two parts occurs at this stage (Fig. 3), and each part develops the adult form (Fig. 4). In chain-formation, the two parts attain the adult form while still united (Fig. 5), and separation of the parts is either much delayed or permanently suppressed. In normal fission, the processes of transverse constriction and separation of parts take about twenty minutes; in chain-formation, the two parts remain together several hours or permanently. The parts of the chains usually remained united several hours in the work of the Chattons, but the union was permanent in most, if not all, of the chains produced in the present work. The Chattons also found that some chains developed into three or four parts as a consequence of a second incompleted fission, and that all chains possessed elongated alimentary vacuoles. In the present work, the alimentary vacuoles of chains retained the normal spherical shape and no chains of three or four parts were observed. In all chains isolated for observation, the junction between the two parts widened and their aboral surfaces gradually bent more and more towards each other (Fig. 6). This process continued, with increasing fusion of the two aboral surfaces (Fig. 7), until their entire lengths were completely fused (Fig. 8). Such individuals thus contained two complete sets of internal structures arranged along parallel axes, but with opposite polarity: the former anterior aboral surface of each part was fused to the former posterior aboral surface of the other part. From such heteropolar double animals, multiple monsters of irregular form (Fig. 9) arose as a consequence of further incomplete fissions: sets of internal structures (nuclei, oral grooves, etc.) multiplied, but the new sets did not separate as new individuals. The usual fate of such irregular multiple monsters was either to die or to break up into smaller irregular monsters (Fig. 10). Occasionally, however, the multiple monsters produced apparently normal individuals (Fig. 11). These gave rise to normal descendants. Less frequently, multiple monsters produced double individuals (Fig. 12) with their two sets of internal structures having identical polarity. The identical polarity of the two parts of these doubles is in contrast to the polarity of the doubles (Fig. 8) earlier formed. The production of such homopolar doubles from multiple monsters was not reported by the Chattons.

The probable mode of origin of normals and homopolar doubles from multiple monsters was indicated by the structure of the monsters. Occasionally, a projection on a monster contained a single set of structures (Fig. 9*a*); fission across such a projection would yield an indi-



FIGS. 1–16. The normal division cycle and the origin of doubles from chains and from "pseudo-conjugants." Figs. 1, 4, 5, 6, 7, 8, 11, 12, 13, and 14 from camera lucida drawings; Figs. 2, 3, 15, and 16 from notebook sketches; Figs. 9 and 10 diagrammatic. Figs. 1 and 11, normal adults; Fig. 2, normal fission nearly completed; Fig. 3, fission just completed; Fig. 4, recent products of fission that have already attained adult form; Fig. 5, chains of two parts, each part having the elongated adult form; Fig. 6, chain with the two parts united over a wider region and forming an obtuse angle; Fig. 7, chain with the two parts united over a large part of their aboral surfaces and forming an acute angle; Fig. 8, heteropolar double; Fig. 9, multiple monster containing a projection (*a*) with one set of structures and another projection (*b*) with two sets of structures lying parallel and having the same polarity; Fig. 10, small multiple monster; Fig. 12, homopolar double; Fig. 13, homopolar double undergoing fission; Fig. 14, homopolar doubles produced by fission of a homopolar double; Fig. 15, "pseudo-conjugant" normal singles united laterally except at anterior end; Fig. 16, "pseudo-conjugant" normal singles united laterally in mid-region.

vidual with normal structure. Rarely, a projection (Fig. 9*b*) contained two sets of structures with the same polarity; fission across such a projection would yield a homopolar double.

Homopolar doubles are of particular importance, from the point of view of the present paper, because they gave rise by fissions to biotypes

of homopolar doubles, as shown in Figs. 13 and 14. The production of multiple monsters from chains and the formation of homopolar doubles by multiple monsters occurred not only once, but many times. All the homopolar doubles studied gave rise to biotypes of doubles like themselves.

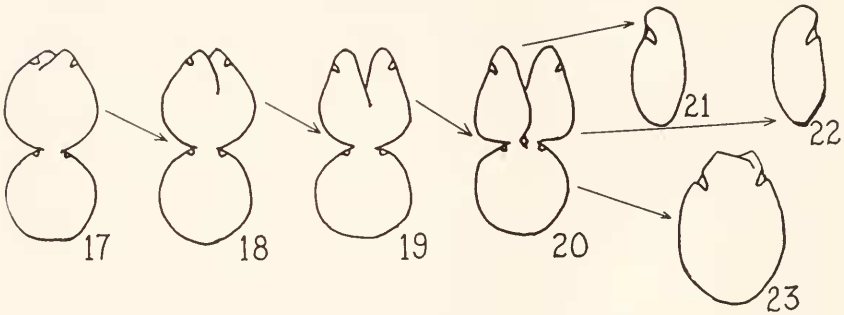
Similar doubles were formed once in an entirely different way. This occurred in a culture of singles left in a Columbia dish a day after most of the colpidia had been removed from it to a finger bowl. (This culture had been fed rye infusion inoculated with *Micrococcus*.) Two individuals (Fig. 15) were found loosely united laterally except at the anterior ends where they did not touch each other; two other individuals were loosely united laterally in the mid-region, but were free at both ends (Fig. 16). These pairs were isolated. Next day, the former had divided into two completely fused pairs; the latter into one completely and one incompletely fused pair. The descendants of each completely fused pair remained united and, like the homopolar doubles produced by multiple monsters, gave rise to biotypes of doubles. These biotypes were kept for three months and, as far as could be judged, could have been kept indefinitely. Thus, in addition to their origin from multiple monsters, biotypes of homopolar doubles arose by lateral fusion of two singles. The superficial resemblance of this fusion to conjugation comes to mind at once, but there are two things that should be remembered in this connection. In the first place, no other phenomenon which might be called normal or abnormal conjugation was ever observed during four years of observation of this species. Secondly, fusions resulting in permanent doubleness are not to be observed in true conjugation. Biotypes of doubles formed by this "pseudo-conjugation" were maintained in mass cultures but not studied farther. In all that follows, it is to be understood that the biotypes of doubles referred to are those that arose from multiple monsters.

V. The Permanence and Stability of Doubles

In both mass and isolation cultures, in both *Achromobacter* and *Micrococcus* fluid, some doubles divided to form two singles and one double instead of two doubles. This came about in the following way. Some doubles (Fig. 17) possessed a small median cleft in the anterior end. In the anterior product of division (Fig. 18) of such an animal, the cleft was usually deeper. Successive anterior products of division (Figs. 19 and 20) contained deeper and deeper clefts until finally one animal (Fig. 20) contained so deep a cleft that the usual transverse fission resulted in the production of two singles (Figs. 21 and 22) from the anterior end, one double (Fig. 23) from the posterior end. The singles and double thus formed reproduced true to their respective types.

This production of singles by doubles raises the question of the permanence of doubles. Will doubles all eventually transform into singles? Or can doubles be maintained indefinitely? As will appear, the answer to these questions depends somewhat on whether mass cultures or isolation cultures are studied. The details of this matter in the two types of cultures will now be set forth.

On April 12, 1930, a mass culture was started of each of eight clones [4] of doubles. These were cultivated in rye infusion inoculated with *Achromobacter,* but the cultures were not kept strictly pure in bacterial content. To each culture fresh fluid was added daily for three or four days, until the original individuals had multiplied to the capacity of the small Columbia dishes. Then the fluid was vigorously sucked up



FIGS. 17–23. Origin of singles from homopolar doubles. Figs. 17, 21, 22, and 23 from camera lucida drawings; Figs. 18, 19, and 20 from notebook sketches. Fig. 17, homopolar double with short median anterior cleft. Fig. 18, double derived from the anterior part of the double shown in Fig. 17; the cleft is deeper. Fig. 19, double derived from anterior part of double shown in Fig. 18; the cleft is still deeper. Fig. 20, double derived from the anterior part of the double shown in Fig. 19; the cleft extends posteriad beyond the fission plane. Figs. 21 and 22, singles derived from anterior part of double shown in Fig. 20. Fig. 23, double derived from posterior part of double shown in Fig. 20.

and expelled from a pipette until the animals were uniformly distributed throughout the fluid and a sample of one or two drops was used to start a new culture in fresh culture fluid. At intervals of three or four days the process of renewal was repeated and observations were made on the proportions of doubles and singles present. At the end of 41 days (May 22), after twelve renewals of the cultures, all clones of doubles except one (clone 2) had transformed into populations consisting exclusively of singles.

[4] The word "clone" is used here in the usual sense of one individual and its vegetative descendants. Arbitrarily, each double produced by a multiple monster has been considered as giving rise to a different clone; this is meant to imply no assumption as to diversity of biotype,—a question treated extensively in Section VI.

A new set of cultures of doubles of these eight clones was started on May 27. In the cultivation of these, the rye infusion was inoculated with *Micrococcus* instead of *Achromobacter*. Renewals of the cultures were made on May 31, June 4, and June 9, before they had to be abandoned. By this time, nine of the cultures had transformed into singles: four of them contained no singles at all, but in the other four 12 per cent to 40 per cent of the colpidia were singles. The average for these four cultures was 26.25 per cent. Comparison with the cultures fed *Achromobacter* after three renewals showed that there were much smaller proportions of singles in the cultures fed *Micrococcus*. Four clones that contained an average of 32.5 per cent singles when fed *Achromobacter* contained no singles when fed *Micrococcus*. The other four cultures averaged 58.75 per cent singles in *Achromobacter,* but only 26.25 per cent singles in *Micrococcus*. This difference in rate of transformation when fed different kinds of bacteria is probably due to a corresponding difference in the rate of multiplication; the colpidia multiplied more rapidly when fed *Achromobacter* than when fed *Micrococcus* (see Table I).

With both types of food, doubles persisted for many generations; hence maintenance of doubleness does not depend on a diet consisting chiefly of *Micrococcus*. However, in both types of food the cultures of doubles gradually contained a larger and larger proportion of singles. In cultures fed *Achromobacter,* all clones (except clone 2) eventually contained only singles. Had the cultures fed *Micrococcus* been observed longer, in all probability they too would have become completely transformed, though this doubtless would have required a longer time than in the cultures fed *Achromobacter*.

It is important to know how this change from doubles to singles took place. Was it a consequence of the death of all doubles or of the transformation of them all into singles? Or was it a consequence of the elimination of doubles in some other way? If the former alternative, then the biotypes of doubles are essentially "Dauermodificationen"; if the latter, they may not be transient biotypes at all. That the latter alternative is the correct one will appear from the records of isolation cultures given below. Meanwhile it is of interest to discover what factors were responsible for the elimination of doubles from mass cultures.

Obviously, an essential factor in this transformation was the production of singles by doubles. If this occurred repeatedly and was not counterbalanced by other factors, it alone would eventually result in the replacement of doubles by singles under the conditions of culti-

TABLE II

Comparison of fission rates of doubles and singles derived from them. Fission rates given in mean number of divisions per line per day.

	PERIOD	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	WEIGHTED MEAN
Clone 3	Number of singles	5	9	11	48	42	39	—
	Means for singles	2.80	2.73	3.11	2.81	3.61	3.59	3.240
	Means for doubles	2.79	2.74	2.90	2.72	3.31	3.33	3.063
	Number of doubles	6	10	12	47	44	45	—
Clone 7	Number of singles	4	—	2	42	39	33	—
	Means for singles	2.69	—	3.00	2.76	3.65	3.41	3.224
	Means for doubles	2.52	—	2.64	2.48	3.11	3.12	2.851
	Number of doubles	6	—	9	46	40	39	—

vation here employed. The doubles fail to double their number at the fissions which yield one double and two singles; but the singles double their number at every fission and the proportion present is continually being increased by the transformation of some doubles into singles. Hence, in the sampling method of culture renewal here employed, eventually too small a proportion of doubles will be present to find a place in the sample. Thus the rate of transformation of a series of cultures would depend on the frequency with which doubles produce singles. This frequency was not ascertained, so that the relative importance of this factor remains unknown.

Other factors, however, become important as soon as some singles have been produced. The subsequent changes in proportion of the two types present must then depend partly on their relative rates of multiplication and partly on their relative rates of mortality.

The fission rates of the singles and doubles of clones 3 and 7 are given in Table II. In clone 3, the singles reproduced 0.1 to 0.3 fissions per day more rapidly than the doubles in all periods except the first two. In these two periods there was practically no difference (0.01 fission per day) between the means for the two groups. However, there were fewer (ten or less) lines in each group during these periods than in any of the later ones; hence, the comparisons during these periods are correspondingly less valuable than during the other periods in which the difference between the two groups was well marked. In clone 7, there can be no doubt that the singles reproduced more rapidly than the doubles. The difference is well marked in all periods for which comparisons are available, even in those in which but few lines were compared. It can be said, therefore, that certainly in clone 7, and very probably in clone 3, the singles reproduced more rapidly than the doubles.

The mortality rates for these groups are given in Table III. With a negligible exception in clone 3, during the period July 4–7, the mortality of singles in both clones 3 and 7 is consistently greater than the mortality of the corresponding doubles. In both clones the mortality rate for the total of all periods is over twice as great among the singles as among the corresponding doubles.

Therefore, in the series of mass cultures of doubles, the gradual change of the proportions of doubles and singles present was not brought about by differential mortality,—indeed was retarded by it; but was due to the continual production of singles by doubles and to the faster reproduction of the singles thus produced. That the death of all doubles or the transformation of them all into singles played no rôle in their extinction is demonstrated by the following account of isolation cultures of doubles.

On April 22, 1930, 48 lines of doubles were isolated in concavities on culture slides. Among these lines eight clones were represented by six lines each. Each line was cultivated in isolation until June 2, 1930. During these 42 days of culture, every clone continued to maintain itself as doubles; no clone died out or transformed completely into singles.

TABLE III

Comparison of mortality rates of doubles and singles derived from them

		PERIOD	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	Singles	No. line-days	50	60	192	188	230	720
		No. died	0	1	0	6	9	16
		No. deaths per 100 line-days	0.0	1.7	0.0	3.2	3.9	2.2
	Doubles	No. deaths per 100 line-days	0.0	0.0	0.05	2.1	0.9	1.0
		No. died	0	0	1	4	2	7
		No. line-days	50	60	191	190	234	725
Clone 7	Singles	No. line-days	—	52	186	179	218	635
		No. died	—	9	8	8	16	41
		No. deaths per 100 line-days	—	17.3	4.3	4.5	7.3	6.5
	Doubles	No. deaths per 100 line-days	—	3.3	1.1	4.4	3.1	2.9
		No. died	—	2	2	8	7	19
		No. line-days	—	60	188	183	225	656

Singles appeared occasionally along with the doubles, especially during the early history of the lines; but during the last 18 days of cultivation no singles were produced in any of the lines. This change in the frequency with which singles were produced was probably a consequence of the method of selection followed each day, for each day the line was perpetuated by the most perfectly doubled individual present (that is,

by the one showing the least development of an anterior cleft). The period (42 days) during which these isolation cultures were maintained as doubles was as long as the period (41 days) during which the mass cultures had transformed completely to singles. Further, these same clones of doubles were maintained from April 5 until October 15. Part of this time they were in isolation culture, part of the time in mass culture; in the latter renewals were made by selecting chiefly doubles. As the usual rate of reproduction was three fissions per day, about 582 generations must have passed while the colpidia remained double. At the end of this time the cultures were discontinued, but there was no reason to suppose that the doubles could not have been maintained indefinitely.

The stability of the doubles was further demonstrated by their maintenance of organization through encystment. The one time encystment was observed in four years of close attention to *Colpidium campylum,* it

FIGS. 24 and 25. Both from notebook sketches and measurements made with ocular micrometer. Fig. 24, cyst 164 μ x 85 μ containing three double colpidia, two small and one large. Fig. 25, one of these doubles immediately after excystment.

occurred in a line of double animals, during isolation culture, 34 days (more than 100 generations) after this line originated from a multiple monster. The cyst (Fig. 24) was discovered less than 24 hours after it formed; in it there were three vigorously moving doubles. Of these three, one was large, two small. Within half an hour, the larger animal divided into two. The cyst remained in this condition one more day. On the third day, five animals were present in the cyst; on the fourth day, six animals. On the fifth day, the cyst was opened with a fine glass needle to allow the encysted animals (of which one is shown in Fig. 25) to emerge. Each of these was a double animal; four of them were used to initiate separate lines of descent. Records showed no differences among the emerged animals and their descendants or between the descendants of encysted and non-encysted members of the same clone, so that more detailed studies on this matter were not made. However, the important point is this: in spite of the reorganizations known to occur in cysts of ciliates, the encysted colpidium did not reorganize as a normal single, but emerged from as it had entered the cyst,—with the double organization.

VI. Diversity of Biotype Among Doubles and Among the Singles Produced by Them

The origin of diverse biotypes during vegetative reproduction has been the subject of numerous investigations (see review by Jennings, 1929). To these must be added the present one on *Colpidium,* in which it has been shown (Section IV) that biotypes of doubles originate from a clone of singles under the influence of a special environmental condition (diet including *Micrococcus*). Further (see Section V), some individuals in these biotypes of doubles give rise to new biotypes of singles. We take up in this section the question of whether there arise other biotypic diversities among the doubles and the singles produced by them. Of this question there are several aspects: (1) Do biotypic diversities exist among the different clones of doubles? (2) Do

Table IV

Comparison of fission rates of doubles of clones 3 and 7. Rates given in number of divisions per line per day.

PERIOD		May 1–7	May 8–14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	Weighted mean for 8 periods
Clone 3	No. lines	6	6	6	10	12	47	44	45	
	Fission rate	3.05	3.05	2.79	2.74	2.90	2.72	3.31	3.33	3.064
Clone 7	Fission rate	2.95	2.73	2.52	2.58	2.64	2.48	3.11	3.12	2.835
	No. lines	6	6	6	10	9	46	40	39	
Clone 3 minus Clone 7		0.10	0.32	0.27	0.16	0.26	0.24	0.20	0.21	0.229

biotypic diversities exist among singles derived from different clones of doubles? (3) Do biotypic diversities exist among singles produced independently from the same clone of doubles? (4) Do biotypic diversities exist between singles produced by doubles and singles not descended from doubles? (5) Do biotypic diversities exist among different lines of descent within a clone? These questions will now be taken up in the order mentioned.

(1) *Biotypic diversities among different clones of doubles.*—Eight clones of doubles were compared in (*a*) the rate of decrease in proportion of doubles present in series of mass cultures; (*b*) the rate of multiplication; and (*c*) the rate of mortality.

(*a*) Striking differences in the rate of decrease in proportion of doubles present appeared among the clones cultivated in series of mass cultures, as described in Section V. In the series fed *Achromobacter,* at the end of the period of observation, no doubles remained in any of the eight clones, except in clone 2. In this clone, however, the vast majority of colpidia present were still doubles. Clearly, the rate of decrease in proportion of doubles was less in clone 2 than in any other clone. This was apparent in both types of food cultures and at all stages in the series. For example, after three renewals of the cultures, all colpidia of clone 2 were still double when fed *Micrococcus* and 95 per cent were double when fed *Achromobacter.* But in clone 3 only 60 per cent of the colpidia were double in *Micrococcus* and only 5 per cent in *Achromobacter.* Between these two extremes, in clone 8, 88 per cent of the colpidia were doubles in *Micrococcus,* 60 per cent in *Achromobacter;* and in clone 1, 72 per cent were doubles in *Micrococcus,* 40 per cent in *Achromobacter.* The different clones thus manifested at least four different rates of transformation and these differences between the clones were the same in both types of culture fluid.

(*b*) An extensive comparison was made of the rates of multiplication in clones 3 and 7. Their mean fission rates for eight periods of from four to seven days each are given in Table IV. In all periods clone 3 multiplied more rapidly than clone 7. At the end of the third period, the slowest line of clone 3 was selected to give rise to all later members of the clone; at the same time, the fastest line in clone 7 was selected to give rise to all later members of this clone. As appears in the table, this radical adverse selection in both clones changed neither the direction nor the magnitude of the difference between the two clones. Clone 3, for the eight periods, had a mean rate of 3.064 fissions per line per day; during the same time, clone 7 had a mean rate of 2.835 fissions per line per day. The different periods gave results very similar to the general mean: in five of the eight periods the excess of clone 3 over clone 7 was between 0.20 and 0.27 fission per line per day; in two periods it was below this range (0.10 and 0.16 fission) and in one period above it (0.32 fission). There can be no doubt of the uniform hereditary difference between clones 3 and 7 in fission rate.

(*c*) The rates of mortality were also extensively compared in these two clones (see Table V). In seven periods, extending over a period of 86 days and including records for 54 of these days, the mortality rates were 0.78 deaths per 100 line-days in clone 3 and 2.40 deaths per 100 line-days in clone 7. In no period is the mortality rate of clone 3 higher than that of clone 7. The difference in mortality rates of these

two clones appears constantly through all parts of the experiment and is thus a biotypic diversity.

Among the clones of doubles here compared, clones 2 and 8 arose at different times from one multiple monster and the other six clones arose at different times from another multiple monster. Thus, differences in the rate of decrease in proportion of doubles present in series of mass cultures existed between clones derived from different multiple monsters: clones 2 and 8 as compared with clones 1 and 3; but similar differences also existed between clones derived from the same multiple monster: the rate in clone 2 differing from that in clone 8, and the rate in

TABLE V

Comparison of mortality rates of doubles of clones 3 and 7

	PERIOD	April 22–May 14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	No. line-days	120	48	50	60	191	190	234	893
	No. died	0	0	0	0	1	4	2	7
	No. deaths per 100 line-days	0.0	0.0	0.0	0.0	0.05	2.1	0.9	0.78
Clone 7	No. deaths per 100 line-days	1.7	0.0	0.0	3.3	1.10	4.4	3.1	2.40
	No. deaths	2	0	0	2	2	8	7	21
	No. line-days	120	48	50	60	188	183	225	874

clone 1 differing from that in clone 3. Likewise, differences between clones of doubles (clones 3 and 7) derived from the same multiple monster were found in rate of fission and in rate of mortality.

(2) *Biotypic diversities among singles derived from different clones of doubles.*—Clones 3 and 7 of doubles have just been shown to differ in rate of fission and in rate of mortality. Do the singles produced by these two clones of doubles differ in the same way? One single from each of these two clones of doubles was permitted to give rise to a number of lines and the mean fission rates of these two groups of singles were compared in four periods. The differences found were small and not constant, so that no significance may be attached to them. In mortality rate, however, the situation was different. As appears in Table VI, in all four periods the rate of mortality is greater—usually very

much greater—among the singles of clone 7 than among the singles of clone 3. The rate for the total time is 2.39 deaths per 100 line-days in clone 3 and 6.33 deaths per 100 line-days in clone 7. The rate for clone 7 is thus 2.65 times as great as that for clone 3. In connection with this difference, it is of interest to note (see Table V) that the doubles of clone 7 had a mortality rate 3.08 times as great as the doubles of clone 3.

TABLE VI

Comparison of mortality rates of stocks of singles produced by one single from a clone 3 double and one single from a clone 7 double

	PERIOD	June 4–11	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles from clone 3 double	No. of line-days	42	60	192	188	230	712
	No. of deaths	1	1	0	6	9	17
	No. of deaths per 100 line-days	2.4	1.7	0	3.2	3.9	2.39
Singles from clone 7 double	No. of line-days	44	52	186	179	218	679
	No. of deaths	2	9	8	8	16	43
	No. of deaths per 100 line-days	4.5	17.3	4.3	4.5	7.3	6.33
Rate of clone 7 singles minus rate of clone 3 singles		2.1	15.6	4.3	1.3	3.4	3.94

(3) *Biotypic diversities among singles produced independently from the same clone of doubles.*—As set forth above, singles were produced two at a time from the anterior half of a double animal. One arises on the right side, one on the left (see Fig. 20). Do the two singles of such a pair differ? That there might be a difference in symmetry was suggested to me by Mr. Donald Costello; but I was unable to detect it. However, it was clear that the two singles of a pair differed sometimes, but by no means always, in other respects. These differences occurred when the anterior cleft of the parental double was much shifted from the usual median position. Frequently the narrower part was separated off as a single one division or more before the broader part. The single produced from the narrower part was invariably narrow, pale, and short as compared with the single produced from the broader part or with the singles ordinarily produced from doubles. The fates

of these two different types of singles were frequently observed. The difference in their fates is illustrated by an experiment on 48 lines observed from May 16–19, 1930. In this group, of the seven singles derived from the narrower parts of unequally cleft doubles, the progeny of four died. Among the 41 lines not derived from narrow parts of unequally cleft doubles, the progeny of only two died. Thus 57 per cent of the one group died as compared with only 5 per cent of the other group. Many other observations confirmed the results in these groups, so that there was no doubt of the very much greater mortality among

TABLE VII

Comparison of mortality rates of a group of singles descended from one single produced by a double of clone 7 and one single not descended from doubles.

	PERIOD	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles not descended from doubles	Number of line-days	479	384	376	473	1712
	Number of deaths	5	3	10	8	26
	Number of deaths per 100 line-days	1.0	0.8	2.7	1.7	1.52
Singles from clone 7 double	Number of line-days	52	186	179	218	635
	Number of deaths	9	8	8	16	41
	Number of deaths per 100 line-days	17.3	4.3	4.5	7.3	6.46
Singles of clone 7 minus singles not descended from doubles		16.3	3.5	1.8	5.6	4.94

the descendants of singles derived from the narrower parts of unequally cleft doubles. Whether the singles produced from the ordinary medially cleft doubles at one time were diverse from those produced from a double of the same clone at another time was not investigated.

(4) *Biotypic diversities between singles descended from doubles and singles not descended from doubles.*—Comparisons were made between singles descended from doubles and singles not descended from doubles in rate of fission and in rate of mortality. The differences found in rate of fission were small and not consistent, so that no significance may be attached to them. In rate of mortality, however, the differences were clear (see Table VII). The total difference between the singles not de-

scended from doubles and a group of singles descended from one single produced by a double of clone 7 is very great: the mortality rate of the latter group is 4.25 times as great as that of the former. This difference is clearly manifested in every period and demonstrates a biotypic difference in rate of mortality between these two groups of singles.

(5) *Biotypic diversities within a clone.*—As already set forth, the most striking differentiation into biotypes that occurs within a clone of doubles is its splitting into biotypes of singles and doubles. This occurred repeatedly in all clones of doubles studied. Furthermore, the biotypes of singles so formed within a clone of doubles were not all alike: some were normal singles, others were narrow, pale, and highly inviable. In addition to these biotypic differences, there were indications of other biotypic differences within a clone, among the doubles themselves. In the isolation cultures of doubles maintained from April 22 until June 2, the frequency with which singles were produced changed strikingly. This was probably brought to light by the practice of selecting daily from among the individuals produced during the previous 24 hours in each line, the individual which showed least development of an anterior cleft. In each line this individual was used to perpetuate the line and the remaining individuals were discarded. In spite of this method of selection, cleft individuals continued to appear and give rise to singles during the early history of these isolation lines. During April 22–30, singles were produced in 31 of the 48 lines of doubles under cultivation. In some lines they appeared more than once. The product of the number of lines in which they appeared by the number of days on which they appeared gives a measure of their frequency of production. This product was 63 line-days, yielding an interval of 6.86 line-days between successive productions of singles. These figures are in striking contrast to those obtained for the same lines during the period May 16–June 2. Not one single was produced during these 864 line-days. In order to bring to light such a great change in the frequency with which doubles produced singles within the same lines of descent, there would have to be genetic differences in frequency of single production among the doubles of each clone. Such a conclusion seems required by the evidence.

Attempts to isolate by selection biotypic differences in rate of fission within clones of doubles, within clones of singles, and within clones of singles descended from doubles were all fruitless. The coefficient of variation of fission rate of a clone of doubles (8.38 per cent) was greater than the corresponding coefficients for singles descended from this clone of doubles (6.46 per cent) and for a clone of singles not descended

from doubles (6.66 per cent). But this greater variability of the clone of doubles was probably not an index of the existence of biotypic diversities in fission rate within this clone, because extreme selection did not result in the isolation of sub-clones with diverse fission rates.

VII. Discussion

The racial effects of environmental conditions found here in *Colpidium* bear a striking parallel in many respects to the relations previously found in the rhabdocoel turbellarian, *Stenostomum incaudatum* (Sonneborn, 1930*b*). As in *Colpidium*, so in *Stenostomum*, special environmental conditions induced abnormalities in reproduction resulting in the formation of irregular monsters. These, likewise, gave rise to double animals that differed from each other and produced races of doubles differing in the same way. Further, in both *Colpidium* and *Stenostomum* the races of doubles maintained their character after removal from the environment that induced their formation, except that singles were formed whenever a cleft of sufficient extent occurred in the growing region perpendicular to the plane of fission. In both, singles gave rise to races of singles of higher viability than the doubles from which they arose.

The degree of similarity between a protozoan and a flatworm in the effects of environmental conditions on their hereditary characteristics is particularly striking in contrast to the very different results of most similar work on higher organisms. What is the basis of this difference? It seems to be the method of reproduction. In sexual reproduction, change of hereditary characters depends largely on changes in the nature or in the composition of the chromatin. Environmental conditions of special penetrability are required to get at this material, so that but few environmental conditions are effective in altering hereditary characteristics. In asexual reproduction, on the other hand, change of hereditary characters may be brought about without in the least affecting the nature or composition of the chromatin; changes in the composition of components of a larger order are also capable of self-perpetuation—that is, are heritable. Examples of this are the rearrangement of parts in homopolar doubles in *Stenostomum* and *Colpidium*. That changes in the chromatin were not involved in these examples was demonstrated by the fact that when individuals were produced from cleft parts of doubles, these were always singles and gave rise to biotypes of singles. The type of hereditary change involved in the production of biotypes of doubles in *Stenostomum* and *Colpidium* is similar to the type involved in the production of stocks of *Drosophila* in which the two X-chromo-

somes are united or in which translocations, inversions, or reduplications have occurred. All such examples illustrate hereditary changes not due to changes in the nature of the germinal material, but due to changes in the number of units or arrangement of units in the germinal material.

It is remarkable that very diverse environmental conditions acting on such diverse organisms as *Colpidium* and *Stenostomum* should result in similar stable types. It may be that this is another example of the stability of whole multiples, as in polyploids; and that of all the teratological consequences of diverse original stimuli, the whole multiples that result are particularly of the viability requisite for survival and self-perpetuation.

VIII. Summary

In a clone of *Colpidium campylum* (Stokes), a small proportion (less than 1.2 per cent) of the individuals formed chains when cultivated in a rye infusion inoculated with the bacterium *Micrococcus sp.* (probably *sensibilis*), but not when the infusion was inoculated with *Achromobacter sp.* (probably *candicans*). Other factors, one of which possibly was the concentration of colpidia in the culture fluid, affected the proportion of chains formed when the colpidia were fed *Micrococcus*. Chains thus produced went through a series of developments including the formation of heteropolar doubles and multiple monsters, and culminating often in the formation of homopolar doubles of a self-perpetuating, relatively stable sort. Similar biotypes of doubles were also formed once as a result of a "pseudo-conjugation." In clones of homopolar doubles, singles sometimes arose by ordinary transverse fission across a double with a deep median anterior cleft. Consequently, mass cultures begun with doubles eventually contained singles also. As the two types multiplied side by side, the relative proportion of singles gradually increased. When the cultures were regularly renewed by taking a sample of the old culture to start a new one, eventually, after many such renewals, doubles entirely disappeared from the cultures, leaving only singles. The change occurred in both *Achromobacter* and *Micrococcus* fluid, but more rapidly in the former; this was probably due to the more rapid reproduction in that fluid. The change in the mass cultures was not due to dying out of doubles or to the transformation of all of them into singles. In isolation culture, lines of doubles were maintained as long as the period required for doubles to disappear entirely from mass cultures. Furthermore, when doubles were deliberately salvaged at each renewal of culture, they were maintained in cultivation, partly in isolation, partly in mass, for 194 days, during which about 582 generations passed. The disappearance of doubles

from mass cultures in 41 days or less must therefore have been due to other factors than the inability of doubles to live and reproduce their kind. One of these factors was a differential rate of fission: the singles produced by the doubles of one clone multiplied 0.373 fissions per line per day more than the doubles of this clone. On the other hand, differential mortality counteracted this to some extent, for the mortality rate of singles was higher than the mortality rate of the doubles that produced them. The gradual disappearance of doubles in series of mass cultures was therefore due partly to their repeated production of singles and partly to their lower fission rate. The persistence of doubles during nearly 600 generations, by the end of which time no evidence of inability to maintain themselves had yet appeared, indicates that the type could have maintained itself indefinitely, even when the bacterium that led to its formation was absent or present in but very small quantities. Further evidence of stability of organization was the passage of a line of doubles through encystment without loss of the double organization.

The question of whether diverse biotypes existed among the experimentally produced doubles and their descendants was extensively investigated. (1) Different clones of doubles differed (*a*) in the rate at which doubles disappeared from series of mass cultures, (*b*) in rate of multiplication, (*c*) in rate of mortality. (2) Singles derived from diverse clones of doubles differed in rate of mortality to about the same extent as the clones of doubles from which they had been derived. (3) There were two very different kinds of singles derived from the same clone of doubles: the usual kind and a rarer kind formed by transverse fission across an unequally cleft double. The singles formed from the narrower anterior part of these were narrower, paler, and shorter than ordinary singles and had a much higher rate of mortality. (4) Singles not descended from doubles had a lower rate of mortality than singles descended from doubles. (5) Within a clone of doubles there were genetic differences in the frequency with which singles were produced, for long-continued selection within lines of doubles brought to light very great changes in this frequency. Attempts to isolate by selection biotypic diversities in rate of fission within clones of doubles and of singles failed, although the coefficient of variation of fission rate was higher for doubles than for singles.

The general picture of the genetic consequences of environmental action in the ciliate protozoan, *Colpidium campylum,* is strikingly similar to the picture in the rhabdocoel turbellarian, *Stenostomum incaudatum.* The similarity in these and the difference of both from the genetic effects of environmental action in higher organisms were ascribed to the method of reproduction. In asexual reproduction hereditary

changes may arise without altering the nature of the chromatin; they may be due simply to changes in the number of units or arrangement of units in the self-perpetuating parts. The changes induced in *Colpidium* and *Stenostomum* were of this sort and, in this respect, resemble translocations, inversions, and reduplications in *Drosophila*.

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